

# High-throughput sequencing reveals differing immune responses in the intestinal mucosa of two inbred lines afflicted with necrotic enteritis



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## ABSTRACT

We investigated the necrotic enteritis (NE)-induced transcripts of immune-related genes in the intestinal mucosa of two highly inbred White Leghorn chicken lines, line 6.3 and line 7.2, which share the same MHC haplotype and show different levels of NE susceptibility using high-throughput RNA sequencing (RNA-Seq) technology. NE was induced by the previously described co-infection model using *Eimeria maxima* and *Clostridium perfringens*. The RNA-Seq generated over 38 million sequence reads for Marek's disease (MD)-resistant line 6.3 and over 40 million reads for the MD-susceptible line 7.2. Alignment of these sequences with the Gallus gallus genome database revealed the expression of over 29,900 gene transcripts induced by NE in these two lines, among which 7,841 genes were significantly upregulated and 2,919 genes were downregulated in line 6.3 chickens and 6,043 genes were significantly upregulated and 2,764 genes were downregulated in NE-induced line 7.2 compared with their uninfected controls. Analysis of 560 differentially expressed genes (DEGs) using the gene ontology database revealed annotations for 246 biological processes, 215 molecular functions, and 81 cellular components. Among the 53 cytokines and 96 cytokine receptors, 15 cytokines and 29 cytokine receptors were highly expressed in line 6.3, whereas the expression of 15 cytokines and 15 cytokine receptors was higher in line 7.2 than in line 6.3 (fold change  $\geq 2$ ,  $p < 0.01$ ). In a hierarchical cluster analysis of novel mRNAs, the novel mRNA transcriptome showed higher expression in line 6.3 than in line 7.2, which is consistent with the expression profile of immune-related target genes.

In qRT-PCR and RNA-Seq analysis, all the genes examined showed similar responses to NE (correlation coefficient  $R = 0.85\text{--}0.89$ ,  $p < 0.01$ ) in both lines 6.3 and 7.2. This study is the first report describing NE-induced DEGs and novel transcriptomes using RNA-seq data from two inbred chicken lines showing different levels of NE susceptibility. These findings provide important insights into our current knowledge of host-pathogen interaction and the nature of host genes that can serve as NE resistance markers for molecular breeding.

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## 1. Introduction

Necrotic enteritis (NE) is an acute or chronic enterotoxemia caused by *Clostridium perfringens* (*C. perfringens*) where a prior infection with *Eimeria maxima* (*E. maxima*) is a primary risk factor in chickens, turkeys, and ducks (Bannam et al., 2011; Savva et al., 2012; Mot et al., 2013). With the regulatory ban of antibiotic growth promoters, NE which is caused by netB toxin-producing *C. perfringens* type A and to a lesser extent by type C strains (Songer,

1996; Yan et al., 2013), is becoming an important enteric disease in chicken worldwide (Bannam et al., 2011; Savva et al., 2012; Mot et al., 2013) and has been estimated to cost the world poultry industry approximately \$2 billion annually (McReynolds et al., 2004). However, the molecular mechanisms underlying the pathology of NE remain to be determined. High-throughput RNA sequencing (RNA-Seq) is a recently developed approach that uses a massively parallel sequencing strategy to generate transcriptome profiles (Wang et al., 2013) and is considered a revolutionary tool for transcriptomics, as it can absolutely quantify millions of unknown transcripts. It has also shown great analytical power for the identification of transcripts that are differentially expressed in response to different conditions (Wang et al., 2011; Xia et al., 2013).

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In a dual infection model of NE, *E. maxima* facilitates the replication and toxin production of *C. perfringens* and is thus used in conjunction with *C. perfringens* for the development of experimental NE disease models (Lee et al., 2011). To date, many studies have used RNA-seq technology for transcriptome analysis in mammals. However, this is the first time this technology has been used to study host pathogen interaction in NE in the intestinal mucosa using two inbred White Leghorn chickens differing NE susceptibility. In this study, we experimentally induced NE using *E. maxima* and *C. perfringens* to identify differentially expressed mRNA in intestinal mucosa of the chicken lines 6.3 and 7.2 which have been selected for their disparate susceptibility to Marek's Disease (Briles et al., 1977). Our new findings provide further insights into the molecular mechanisms that underline posttranscriptional regulation by mRNA of mucosal tissue in NE-afflicted chickens that will facilitate the development of novel strategies to control NE and the new genes identified in this study will be useful molecular markers for selecting NE resistance in commercial chickens.

## 2. Materials and methods

### 2.1. Experimental animals

The two White Leghorn chicken lines, namely, the MD-resistant line 6.3 and MD-susceptible line 7.2, were obtained from the Avian Disease and Oncology Laboratory (East Lansing, MI, USA) of the Agricultural Research Service of the United States Department of Agriculture (USDA). Chickens were raised in Petersime starter brooder units, provided ad libitum access to water, and maintained under uniform standard management conditions. Experimental and control groups were kept in separate rooms, preventing cross-contamination throughout the course of the experiment.

### 2.2. Experimental NE disease model

The method used to produce NE was previously described (Jang et al., 2012). Chickens were infected with *E. maxima* ( $1.0 \times 10^4$  oocysts/bird) by oral gavage on day 14, which was followed by oral gavage with a field strain of *C. perfringens* strain Del-1 ( $1.0 \times 10^9$  colony forming units [CFU]/bird) after 4 days. For development of NE, the birds were fed a non-medicated commercial basal ration of 17% crude protein from 1 to 18 days of age, and at 18–20 days of age, the feed was replaced with commercial non-medicated feed containing 24% crude protein. All protocols were approved by the Beltsville Area Institutional Animal Care and Use Committee.

### 2.3. Total RNA preparation

The small intestines from five chickens per group were cut longitudinally and briefly washed 3 times with ice-cold Hanks' balanced salt solution (HBSS) containing 100 U/mL penicillin and 100 mg/mL streptomycin (Sigma, St. Louis, MO, USA). The mucosal layer was carefully removed using a cell scraper (Nunc, Thermo Scientific Inc., Waltham, MA, USA). The samples were placed on ice immediately and maintained on ice until the total RNA extraction. Total RNA was isolated using TRizol reagent (Invitrogen, Carlsbad, CA, USA) as described (Hong et al., 2012). RNA concentrations were quantified using a NanoDrop spectrophotometer (NanoDrop Technologies, USA), and the 260/280 nm ratio was confirmed to be between 1.7 and 2.0. The integrity of the total RNA samples was evaluated using the Agilent 2100 (Agilent Technologies, Inc., Santa Clara, CA, USA) and Tecan F2000 (Tecan Group Ltd., Männedorf, Switzerland) devices, and only samples with an RNA integrity

number (RIN) >7.0 and high-quality RNA (28S/18S > 1) were used for the subsequent experiments.

### 2.4. mRNA sequencing and analysis

Reverse transcription was performed and cDNA was synthesized using 5' adaptor forward and 3' adaptor reverse primers. Libraries for Illumina sequencing were constructed from cDNA as described (Trapnell et al., 2010). High-throughput RNA sequencing was performed by Theragen Bio Institute (Suwon, Korea) on an Illumina HiSeq 2000 high-throughput sequencer (Illumina, Inc. San Diego, CA, USA) according to the manufacturer's specifications. RNA-Seq data were analyzed according to the method described (Trapnell et al., 2012). Briefly, reads were mapped to the *Gallus gallus* reference genome (v.4.0) obtained from the University of California, Santa Cruz (UCSC) database (UCSC: <http://genome.ucsc.edu/>) using TopHat v.2.0.3 (<http://tophat.cbcb.umd.edu/>), and Bowtie v.0.12.8 (<http://bowtie-bio.sourceforge.net/index.shtml>) from Illumina iGenomes (<http://support.illumina.com/>). Gene expression values were measured for each gene from the Ensembl database by fragments per kilobase of exon per million mapped reads (FPKM) calculated using Cufflinks v2.0.1 (<http://cufflinks.cbcb.umd.edu/>) (Mortazavi et al., 2008). Differentially expressed genes were considered in a given library when (1) the *p*-value was less than 0.01 and (2) a greater-than-or-equal to 2-fold change in expression across libraries was observed and used to identify the genes differentially expressed between two chicken lines. Subsequently, the differential expression pattern analysis of known mRNA and prediction of novel mRNA were performed using unannotated sRNAs. Gene ontology (GO) terms and annotations were matched on GO terms in the database (<http://www.geneontology.org/>), and the functional enrichment analysis was performed using Blast2GO (v.2.7.1) (<http://www.blast2go.org/>). Further, significantly differed genes from the corresponding library were searched against the Kyoto Encyclopedia of Genes and Genomes (KEGG) database to determine the pathways using DAVID Bioinformatics Resources version 6.7, NIAID/NIH (<http://david.abcc.ncifcrf.gov/tools.jsp>) with *p* < 0.01.

### 2.5. Hierarchical cluster analysis for mRNA

Hierarchical cluster analysis was performed for mRNAs using Cluster version 4.49 software (<http://www.bram.org/serf/Clusters.php>) and Java Treeview software (<http://sourceforge.net/projects/jtreeview/files/>). Intestinal samples of lines 6.3 and 7.2 were compared as treatment and control samples, respectively. Cluster map analysis of genes was performed using the Euclidean distance. The *p* values were calculated using the right-tailed Fisher's exact test.

### 2.6. cDNA synthesis

For analysis of mRNA gene expression levels, 5  $\mu$ g of total RNA was treated with 1.0 unit of DNase I and 1.0  $\mu$ L of 10  $\times$  reaction buffer (Thermo Scientific, Waltham, MA, USA), and incubated for 30 min at 37 °C. Subsequently, to inactivate DNase I, 1.0  $\mu$ L of 50 mM EDTA was added and the mixture was heated to 65 °C for 10 min. RNA was reverse-transcribed using the Maxima First Strand cDNA Synthesis Kit (Thermo Scientific, Waltham, MA, USA) according to the manufacturer's recommendations. Briefly, 5.0  $\mu$ g of RNA was combined with 0.2  $\mu$ g of oligo (dT)<sub>18</sub> primer and RNase-free water to give a total volume of 12.0  $\mu$ L. Then, 4.0  $\mu$ L of 5  $\times$  reaction buffer, 20 units of Ribolock RNase Inhibitor, 10 mM dNTP mix, and 200 units of RevertAid M-MuLV reverse transcriptase were added. The mixture was incubated at 42 °C for 60 min and heated at 70 °C for 5 min to terminate the reaction. After cDNA synthesis, the gene

expression profile was determined using an equivalent amount of cDNA.

### 2.7. Quantitative real-time PCR (*qRT-PCR*)

All real-time PCR primers used in this study for cytokine genes were designed using Lasergene software (DNASTAR Inc. Madison, WI, USA) and synthesized by Genotech Corp. (Daejeon, South Korea). Details for the primers are given in supplementary Table S1. Cytokine gene expression was quantified using standard curves that were generated using  $\log_{10}$  diluted cDNA from individual total RNA samples as described (Hong et al., 2012). cDNA (100 ng) was added into a reaction mix including 10  $\mu$ L of 2  $\times$  Power SYBR Green Master Mix (Roche, Indianapolis, USA), 0.5  $\mu$ L of each primer, and RNase-free water to a total volume of 20  $\mu$ L. Real-time PCR was performed using a LightCycler 96 system (Roche, IN, USA) with the following standard cycling program: pre-incubation at 95 °C for 10 min, followed by 45 cycles of 95 °C for 10 s, 52 °C for 30 s, and 72 °C for 30 s.

### 2.8. Statistical analysis

Statistical analyses were performed using IBM SPSS software (SPSS 20.0 for Windows, Chicago, IL, USA). The data are expressed as the mean values  $\pm$  SD for each group ( $N=5$ ) and were compared between groups using Student's *t*-test. Statistical significance was defined as  $p < 0.05$  and  $p < 0.01$ .

## 3. Results

### 3.1. Data set and alignment of RNA sequence

Biological replicates ( $n=5$ ) were pooled to generate representative samples for deep sequencing analysis. These RNA-Seq libraries provided a total of approximately 38.8 and 40.2 million sequence reads for the MD-resistant line 6.3 and MD-susceptible line 7.2, respectively. Of the total reads, 72.52% and 79.63% were successfully mapped on the chicken genome in line 6.3 and line 7.2, respectively. In each group, 53.84% and 59.02% properly paired for line 6.3 and line 7.2, respectively. Among these reads, 57.08% and 50.74% were mapped to the intergenic/exon-intron regions in the intestinal mucosa of lines 7.2 and line 6.3, respectively (Table S2).

Expression levels were measured as fragments per kilobase of exon per million mapped reads (FPKM) (Trapnell et al., 2010), and the expression level for each gene was the sum of the FPKM values of its isoforms. These fragments or reads were used to measure levels of gene expression and to identify novel splice variants of genes. We detected approximately 30,000 mRNA genes, comprising approximately 10% known genes, 51% novel genes, and 39% known + new transcripts genes among all samples. Among the total genes with novel mRNA, the number of individual genes with  $p < 0.01$  and fold change  $\geq 2$  was generally higher in line 6.3 (887 novel genes) than in line 7.2 (873 novel genes) (Unpublished data).

To evaluate the quality of RNA-Seq data, several quality control analyses were performed. In this study, we used gene coverage to evaluate the RNA-seq results. Gene coverage is defined as the percentage of a gene covered by reads and is equal to the ratio of the base number in a gene covered by unique mapping reads to the total number of bases of that gene (Fig. S1). The distribution of distinct reads over different read abundance categories showed similar patterns for all four RNA-Seq libraries. In chicken line 6.3, the read coverage was more than 90% of the chicken genome for 63% of the read sequences in NE-induced chickens and 61% of the read sequences in uninfected control chickens. In contrast, in chicken

line 7.2, for 65% of read sequences in both NE-afflicted and uninfected control chickens the read coverage was more than 90% to chicken genome.

### 3.2. Gene ontology analysis of the differentially expressed genes (DEGs) in the two chicken lines

For a better understanding of the range of genes involved in the response to the oral infection of *E. maxima* followed by *C. perfringens*, classes of DEGs were determined using gene ontology (GO) analysis. Blast2GO software (v.2.7.1) identified the DEGs and novel transcripts in the NE and uninfected control groups. Among the 560 DEGs, 246 were associated with biological processes, 215 with molecular functions, and 81 with cellular components. Overall, the number of genes upregulated in each term was higher in chicken line 6.3 than in chicken line 7.2 (Table S3A–C).

In the intestinal mucosa of line 6.3, most of the DEGs fell into the subcategories protein binding, membrane, and small molecule metabolic process under the major groups of molecular function, cellular component, and biological process, respectively. In the intestinal mucosa of line 7.2, most of the DEGs fell into the subcategories catalytic activity, membrane, and transport under the major groups of molecular function, cellular component, and biological process, respectively.

### 3.3. Identification of differentially expressed genes between line 6.3 and line 7.2

In this study, FPKM values were used to compare differential mRNA expression levels between chicken line 6.3 and chicken line 7.2. We first analyzed the DEG profiles in chicken line 6.3 and line 7.2 using RNA-sequencing and then found that 7,841 genes were significantly upregulated and 2,919 genes were downregulated in NE-afflicted chicken line 6.3 compared to uninfected control chicken line 6.3, with  $p < 0.01$  and fold change  $\geq 2$ . In addition, 6,043 genes were significantly upregulated and 2,764 genes were downregulated in NE-afflicted line 7.2 compared with the uninfected control, with  $p < 0.01$  and fold change  $\geq 2$ . In addition, a comparison with both NE-afflicted chickens of the gene expression profiles on the intestinal mucosa, 2,707 genes significantly expressed between two lines. Of the 2,707 genes, 1,285 genes were abundantly expressed in chicken line 7.2 than line 6.3 and 1,422 genes were highly expressed in line 6.3 with  $p < 0.01$  and fold change  $\geq 2$  (Table S2).

To understand the functions of the DEGs, we mapped them using the KEGG database for signaling pathways analysis based on using DAVID Bioinformatics Resources version 6.7 with  $p < 0.01$ . In KEGG pathway mapping, 2,851 genes could be assigned gene identification number with the National Center for Biotechnology Information (NCBI) Gene ID. Among them, 1,762 in chicken line 6.3 and 1,970 DEGs in line 7.2 mapped into various pathways at the Kyoto Encyclopedia of Genes and Genomes databases (<http://www.genome.jp/kegg/>), respectively (Unpublished data). The top 16 most abundant differentially expressed signaling pathways from each group are listed in Table 1. Specifically, the cluster for MAPK signaling pathway, Endocytosis signaling pathway, Cytokine–cytokine receptor interaction, JAK-STAT signaling pathway, Toll like receptor pathway and TGF-beta signaling pathway represented the largest group in both lines, which agreed with the notion that response to exchange the environment, an activating stimulus.

Second, we explored the use of RNA-seq to identify spliced transcripts generated by novel splicing events of known annotated exons using an approach similar to TopHat. Hierarchical clustering was used to analyze 91 novel genes of the intestinal mucosa that were differentially regulated between the two chicken lines

**Table 1**

Top 16 most abundant differentially expressed signaling pathways.

ID	Pathway	Line 6.3		Line 7.2	
		DEGs	p-value	DEGs	p-value
gga04010	MAPK signaling pathway	91	0.0018	61	3.9E-3
gga04060	Cytokine-cytokine receptor interaction	70	0.00045	49	0.0013
gga04144	Endocytosis pathway	75	0.000014	57	0.000043
gga04630	Jak-STAT signaling pathway	61	0.0044	41	0.0028
gga04620	Toll like receptor pathway	57	0.0049	34	0.0032
gga04350	TGF-beta signaling pathway	50	0.0034	24	2.3E-3
gga04110	Cell cycle	34	0.0028	23	9.9E-3
gga04020	Calcium signaling pathway	33	0.00034	29	5.7E-08
gga03320	PPAR signaling pathway	32	0.0033	24	1.0E-3
gga04370	VEGF signaling pathway	31	0.0011	22	0.0015
gga04510	Focal adhesion	27	0.0023	16	0.0024
gga04672	Intestinal immune network for IgA production	26	0.0042	15	0.0024
gga04910	Insulin signaling pathway	24	0.0039	15	0.0012
gga04512	ECM-receptor interaction	23	0.00156	10	0.0028
gga04514	Cell adhesion molecules (CAMs)	22	0.0042	13	0.0038
gga04310	Wnt signaling pathway	21	0.0035	14	0.0046

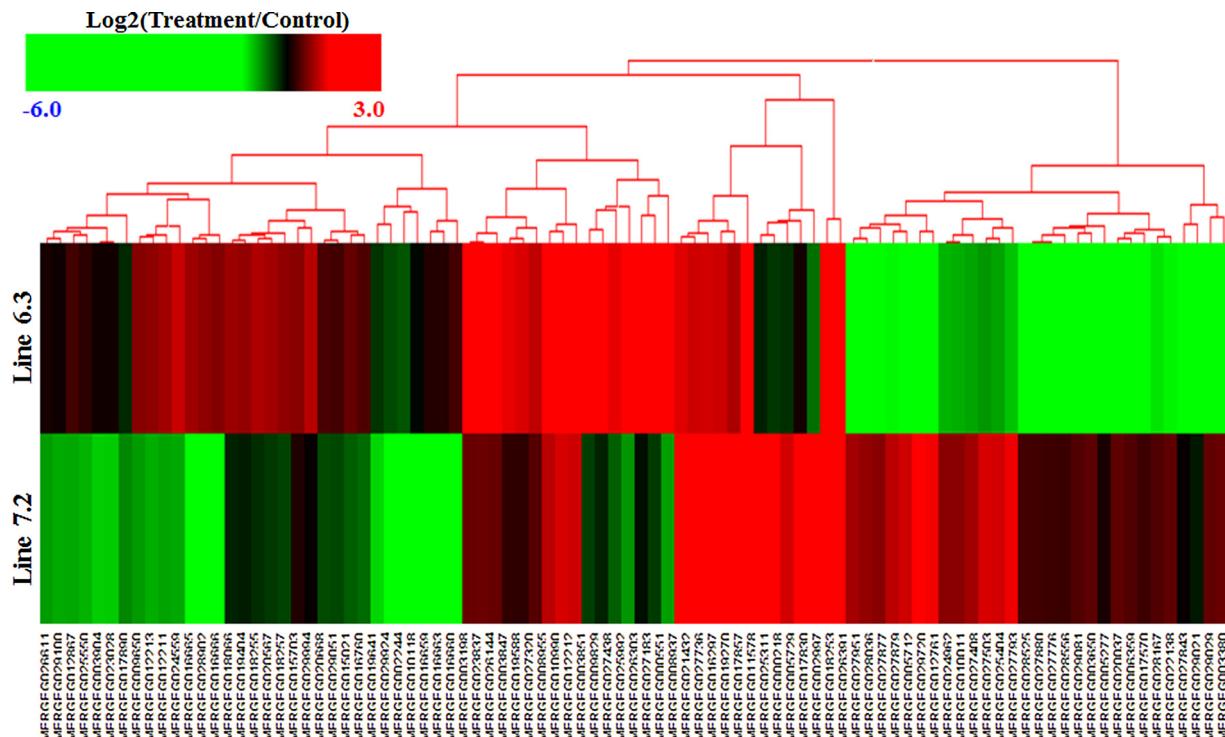
to identify 40 downregulated and 51 upregulated genes in the line 6.3 relative to line 7.2, and vice versa. When the treatment condition was compared with the control in line 6.3, 43 upregulated genes and 11 downregulated genes were identified. In contrast, line 7.2 showed 11 upregulated genes and 27 downregulated genes, respectively (Fig. 1 and Table S4).

Third, we analyzed RNA-seq data to identify immune-related genes, which are biologically important for the host response to antigens. Based on the differences in genetic background between line 6.3 and line 7.2, it was expected that some immune-related genes would be differentially expressed between the two inbred lines that show different levels of NE susceptibility. The results showed that 66 immune-related genes were more highly expressed in line 6.3 than in line 7.2, whereas 56 immune-related genes were

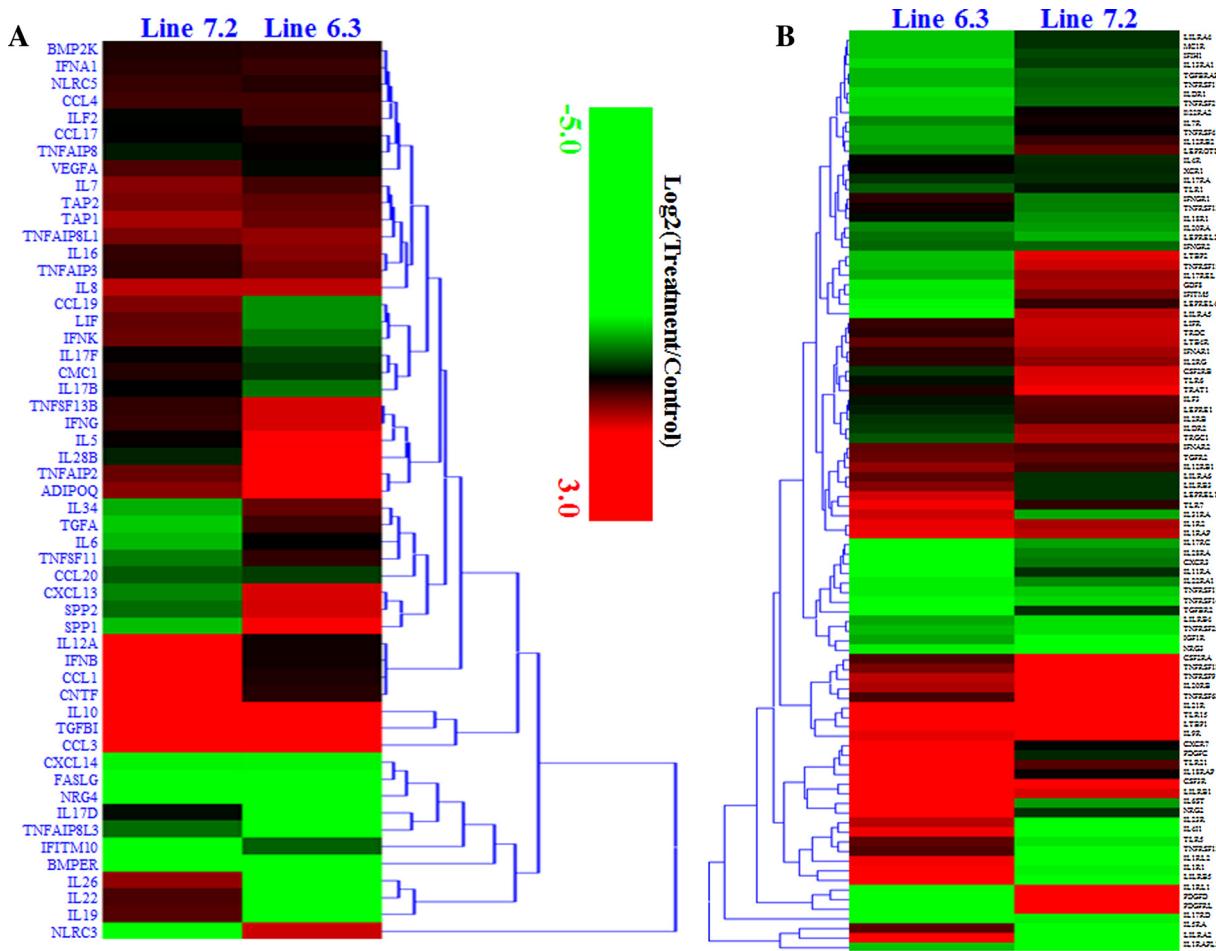
more highly expressed in line 7.2 than in line 6.3 with  $p < 0.01$ , fold change  $\geq 2$  (Table S5).

### 3.4. Innate immune responses in NE-affected chickens

Transcriptome analysis for 53 cytokines and 96 cytokine receptors presented as the NE-induced fold change in comparison with the uninfected control for each chicken line is shown in Table S6. The expression of 7 cytokines (IL-10, IL-12A, CCL1, etc.) significantly increased by 2.48- to 15.23-fold (Fig. 2A and Table S6) and that of 13 cytokine receptor genes (IL-1RL1, IL-21R, IL-20RB, etc.) was also upregulated by 2.0- to 4.56-fold in chicken line 7.2 with  $p < 0.01$  and fold change  $\geq 2$  (Fig. 2B and Table S6). In contrast, 6 growth factor genes (NLRC3, BMPER, IFITM10, etc.) and 6 receptor genes (IL-5RA,



**Fig. 1.** Cluster map analysis of 91 novel genes in the intestinal mucosa in the two chicken lines, MD-resistant line 6.3 and MD-susceptible line 7.2, identified using Euclidean distances. Red and green indicate that the novel mRNA has a significantly higher or lower expression level, respectively. Each row in this figure shown one novel mRNA, and each column shows once sample pair (intestinal mucosa in line 6.3 and line 7.2), with significant expression at  $p < 0.01$  and fold change  $\geq 2$ . (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)



**Fig. 2.** Hierarchical clusters of 53 cytokine (A) and 96 cytokine receptors genes (B) responsive to *E. maxima* and *C. perfringens* coinfection in the intestinal mucosa of two chicken lines (MD-resistant line 6.3 and MD-susceptible line 7.2) were based on Euclidean distance correlation analyses. The genes included here showed significant differences in gene expression ( $p < 0.01$ , fold change  $\geq 2$ ). The genes shown in red were upregulated and those in green were downregulated. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

IL-1RAPL2, IL-1RL2, etc.) were significantly downregulated by 2.0- to 9.76-fold and 2.1- to 7.64-fold, respectively, in chicken line 7.2 with  $p < 0.01$  and fold change  $\geq 2$  (Fig. 2A, B and Table S6).

Similarly, we found that the expression of 8 cytokines (IL-5, IL-10, IL-28B, etc.) was markedly increased by 2.03- to 7.15-fold (Fig. 2A and Table S6) and that of 18 cytokine receptors (IL-18RAP, IL-1R1, IL-1RAP, etc.) was significantly increased by 2.03- to 4.69-fold in chicken line 6.3 ( $p < 0.01$ , fold change  $\geq 2$ ) (Fig. 2B and Table S6). In contrast, the expression of 9 growth factors (IL-22, IL-26, IL-19, etc.) markedly decreased by 2.6- to 7.48-fold and only expression of the IL-17RD receptor gene decreased by 3.67 fold in line 6.3 at  $p < 0.01$  (Fig. 2A, B and Table S6). When cytokine genes were compared between chicken line 6.3 and line 7.2, among the 53 cytokines and 96 cytokine receptors identified in the transcriptome analysis, 15 cytokines (IL-10, IL-28B, IL-34, etc.) and 29 cytokine receptors (IL-18R1, IL-18RAP, IL-1R1, etc.) were highly expressed in line 6.3. Similarly, 15 cytokines (IL-12A, IL-17B, IL-17D, etc.) and 15 cytokine receptors (IL-17RD, IL-1RL1, IL-20RB, etc.) were highly expressed in line 7.2 (Fig. 2A, B and Table S6).

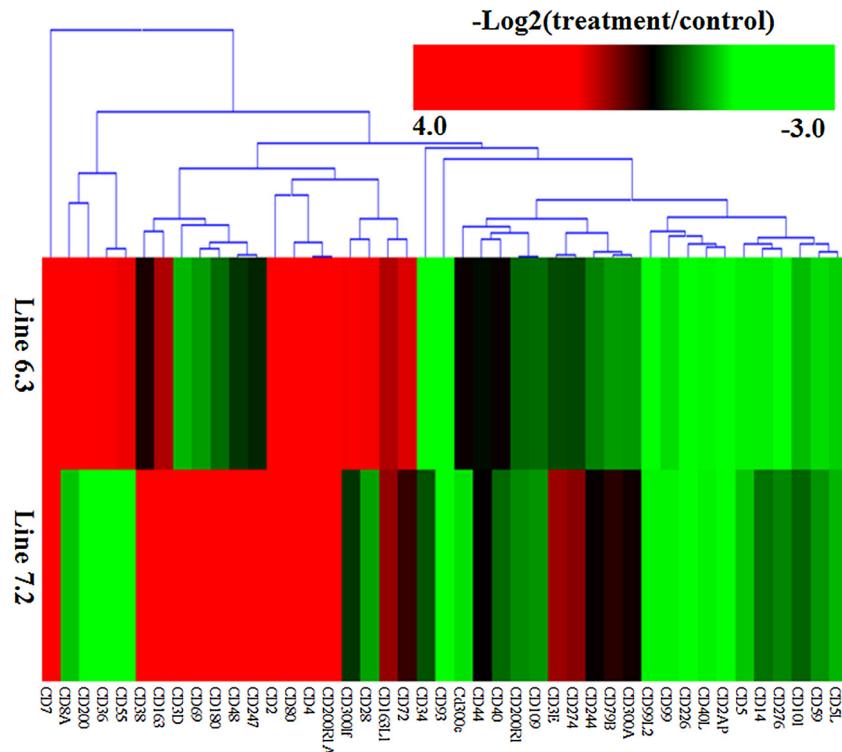
The interleukin 1 (IL1) family comprises inflammatory cytokines produced by activated macrophages (Martin et al., 2013). Among the IL-1 family members, the expression of 4 IL-1 receptors (IL-1R1, IL-1RAP, IL-1RL2, and IL-18RAP) was upregulated in chicken line 6.3 by 2.10 to 3.16 fold. In contrast, IL-1RAP and IL-18RAP expression was upregulated by 0.28 and 1.23 fold, respectively, in chicken line 7.2. Moreover, IL-1R1 and IL-1RL2 expression was downregulated

by 1.43 and 3.29 fold, respectively, in chicken line 7.2. Interleukin 1 receptor-like 1 (IL-1RL1) was upregulated in line 7.2 and downregulated in line 6.3, whereas IL-1 receptor associate and IL-18 receptor 1 were significantly downregulated by 0.49 and 4.92 fold in line 7.2, respectively. Interleukin 1 receptor 2 (IL-1R2) expression was significantly higher in the NE-afflicted groups than in the uninfected control groups in both chicken lines. IFN $\alpha$  and IFN $\gamma$  were highly expressed in line 6.3 (Fig. 2A, B and Table S6).

In addition, we investigated the gene expression patterns of 44 CD molecules in the intestinal mucosa of NE-afflicted chickens in both lines. Transcriptional analysis of the 44 CD genes indicated that 12 CD genes (CD2, CD200, CD200R1A, etc.) were markedly upregulated by 2.1- to 4.6-fold and only expression of the CD34 gene was decreased by 2.18-fold in chicken line 6.3 compared with the uninfected control ( $p < 0.01$  and fold change  $\geq 2$ ). In contrast, 12 CD genes (CD163L1, CD2, CD200R1A, etc.) were significantly upregulated by 2.0- to 5.04-fold and only CD99 was significantly downregulated by 2.11-fold in chicken line 7.2 compared to the uninfected control with  $p < 0.01$  and a fold change  $\geq 2$  (Fig. 3 and Table S7).

### *3.5. Discovery of novel mRNA transcripts*

To explore the use of RNA-seq in identifying spliced transcripts generated by novel splicing events in known annotated exons, the TopHat algorithm pairs candidate exons and evaluates the



**Fig. 3.** Expression of 44 CD marker genes in NE-afflicted chicken lines. The heatmap was generated from a hierarchical analysis of the 44 CD marker genes that showed significant changes in the NE-afflicted chicken lines. The genes included here showed significant differences in gene expression ( $p < 0.01$ , fold change  $\geq 2$ ) in at least one experiment. The genes shown in red were upregulated expression and those in green were downregulated in the two NE-induced chicken lines. Hierarchical clusters of genes and samples were based on Pearson's correlation analyses. (For interpretation of the references to color in this figure legend, the reader is referred to the web version of this article.)

alignment of reads to such candidates (Trapnell et al., 2009). In this study, we detected 29,998 mRNA genes with 15,418 novel genes. Among the total mRNA novel genes, 2,709 novel genes were upregulated in chicken line 6.3, and 1,753 novel genes were upregulated in chicken line 7.2 when compared to uninfected control lines. Moreover, the number of novel individual genes differentially expressed with  $p < 0.01$  and fold change  $\geq 2$  was generally higher in line 6.3 (887 novel genes) than in line 7.2 (873 novel genes) afflicted with NE (Unpublished data).

The results of Euclidean distance analyses using hierarchical clustering for 91 novel genes in the intestinal mucosa in the two lines are shown in Fig. 1 (Table S4). The red color indicates the novel mRNA which is more highly expressed in the NE group than in the uninfected control and the green color indicates the novel mRNA that is more highly expressed in the control than in the NE group. Compared with the control chickens, 53 novel genes in line 6.3 and 46 novel genes in line 7.2 were differentially regulated by NE ( $p < 0.01$ , fold change  $\geq 2$ ). Fig. 1 shows that 51 novel genes whose expression is higher in line 6.3 than in line 7.2. Several genes of interest were identified in the novel contigs, many of which are associated with the response to stress, cellular processes, binding, membrane function, and ATP activity. Several of these transcripts are involved specifically in the immune response, as shown in Table S4.

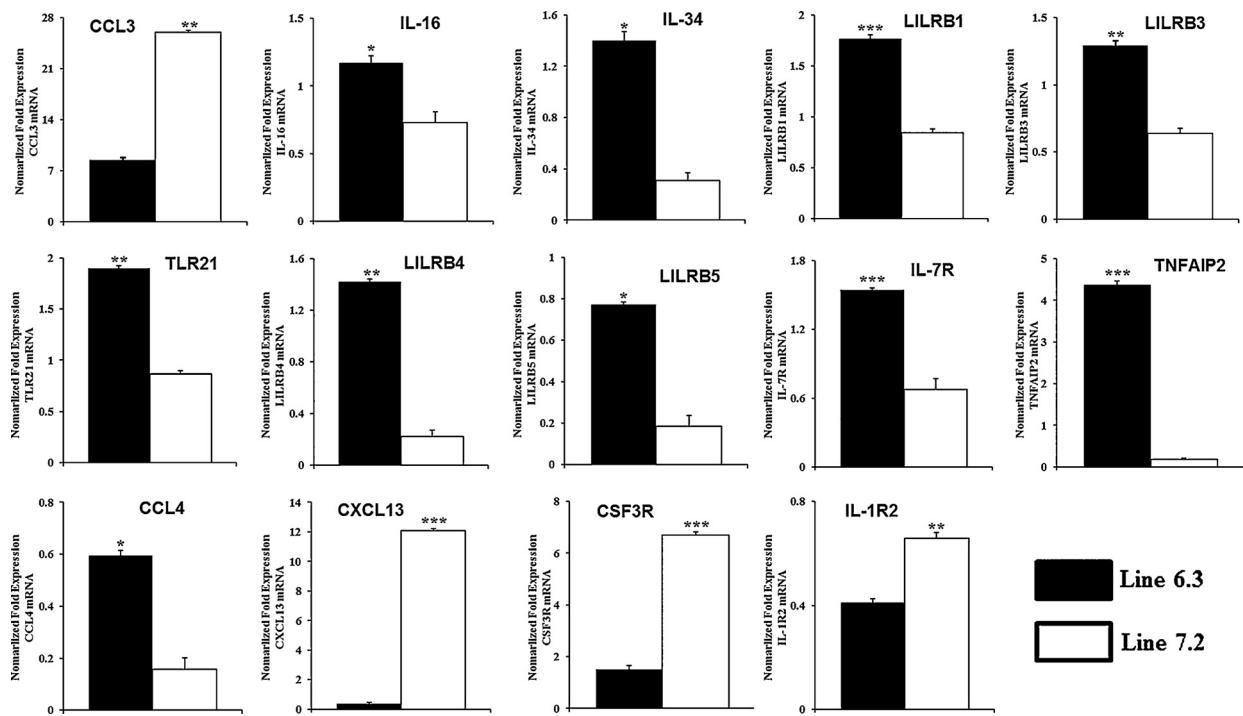
### 3.6. Quantitative real-time RT-PCR

According to the fold-change values determined in the RNA-seq analysis, 14 DEGs showed significant differences in expression on the intestinal mucosa of lines 6.3 and 7.2 (Table S1). We performed real-time qPCR to analyze the expression of 14 DEGs in the intestinal mucosa of chicken lines 6.3 and 7.2 compared with

the respective uninfected controls. Gene expression changes were compared between the two chicken lines by quantitative real-time RT-PCR as shown in [Figs. 4 and S2](#). CCL3 and CSF3R mRNA expression increased in both chicken lines, particularly in line 7.2. Coinfection with *E. maxima* and *C. perfringens* increased CXCL13 mRNA expression in chicken line 7.2 by 12.08 fold ( $p < 0.01$ ). In contrast, NE-afflicted chickens showed a small increase of CCL4, LILRB5, and IL-1R2 mRNA expression in both chicken lines ([Figs. 4 and S2](#)). Most other cytokines and cytokine receptors such as TLR21, LILRB1, LILRB3, LILRB4, IL-34, IL-7R, and TNFAIP2 showed increased expression in both chicken lines and were more highly expressed in chicken line 6.3 than in chicken line 7.2 ([Figs. 4 and S2](#)). In addition, the expression of IL-16 mRNA in the two chicken lines was higher in the NE-affected group than in the uninfected control group ([Figs. 4 and S2](#)). When we compared the gene expression results from real-time qPCR and RNA-Seq, the expression trends were consistent for all transcripts in both analyses with a correlation coefficient of  $R^2 = 0.85$  for chicken line 6.3 and  $R^2 = 0.89$  for chicken line 7.2 as shown in [Fig. 5](#).

## 4. Discussion

In this study, we first evaluated the effect of coinfecting two chicken lines, line 6.3 and 7.2 which show different levels of NE susceptibility ([Jang et al., 2013](#)) with *E. maxima* and *C. perfringens* on intestinal gene expression using high-throughput mRNA sequencing (RNA-seq) technology. We generated draft sequences from two genetically different chicken lines with disparate MD phenotypes. In total, 29,998 mRNA genes were built using alternatively spliced transcripts. Identity of these differentially expression genes between these two lines are important to obtain clues for the protective immune response to NE-causative pathogens. The

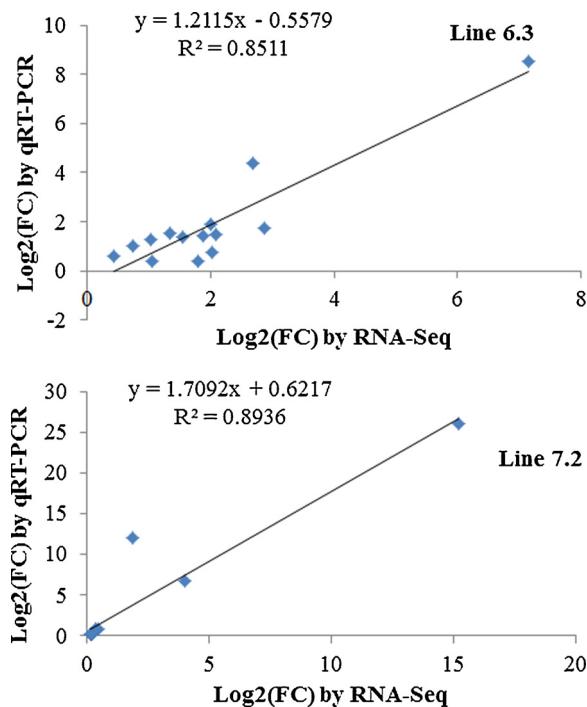


**Fig. 4.** Expression profile of cytokine genes in the intestinal mucosa of two genetically disparate chicken lines. Chickens were orally infected with  $1.0 \times 10^4$  oocysts of *E. maxima* on day 14, followed by *C. perfringens* infection with  $1.0 \times 10^9$  CFU 4 days later. The intestinal mucosa was isolated from chickens at 2 days post C.P., and the transcriptional level was determined by quantitative RT-PCR. Data are expressed as mRNA levels normalized against the GAPDH mRNA level with triplicate determination performed with pooled samples from five chickens. Significant differences in mRNA expression levels between line 6.3 and line 7.2 are indicated as follows: \*  $p < 0.05$ , \*\*  $p < 0.01$ , and \*\*\*  $p < 0.001$ . Error bars indicate SE of technical replicates that were performed in triplicate.

number of aligned reads increased with sequencing depth; however, with read depths greater than 100 million, the percentages remained constant. In contrast, the percentage of aligned reads that map unambiguously to the genome was constant at 84% for all sequencing depths (Toung et al., 2011). More significant DEGs

were obtained in line 6.3 than in line 7.2, that is, 1,285 genes were abundantly expressed in chicken line 7.2 and 1,422 genes were highly expressed in line 6.3. In addition, the number of upregulated genes in NE-infected line 6.3 was higher than that in NE-induced line 7.2 when compared to the uninfected control: 7,841 genes in line 6.3 and 6,043 genes in line 7.2. Moreover, the number of downregulated genes in NE-induced chicken line 6.3 was also higher than that in NE-induced chicken line 7.2 (2,919 genes in line 6.3 vs. 2,764 genes in line 7.2). There was a large difference in the number of DEGs depending on whether the RNA sequence was based on reference-based assembly. In this study, 13,570 of the 14,479 known genes were annotated and differentially expressed. These genes were deemed unique based on the protein identification code of the UniProt ID, suggesting that other factors may contribute to this difference. As expected, based on the proportion of differentially expressed genes, the number of genes enriched the GO biological process was also different between up- and downregulated genes. The GO functional enrichment analysis thus suggests different functions between the two chicken lines.

TopHat algorithm mapping to the chicken reference genome was used to find novel candidate genes. We detected many transcripts with novel splicing among all detected transcripts classified as intergenic regions of intron/exon, complete match, novel or unknown. A total of 15,418 novel splice variants (51% of the consensus sequences) have been detected in our samples. The large number of novel splice variants identified may reflect a large number of false-positive junctions or may indeed reflect a considerable number of hitherto unannotated isoforms in the chicken transcriptome. To further assess the false positive rate, we determined the number of novel genes that matched junctions reported in the EST data. Even though line 6.3 had a substantially larger number of novel genes than line 7.2, our findings provide information on the regulation of genes in both NE-induced chicken lines.



**Fig. 5.** Significant correlations between expressions of qRT-PCR and RNA-Seq in intestinal mucosa of NE-afflicted chicken line 6.3 and line 7.2.

The functional classification of the novel transcripts identified using RNA-seq was highly diverse, with a large proportion involved in the immune response, transport, developmental processes, the stress response, and cell adhesion. In addition, many of these transcripts have been shown to be involved specifically in the immune response, such as those involved in the mitogen activated protein kinase (MAPK) signaling pathway, JAK-STAT signaling pathway, TGF- $\beta$  signaling pathway, and toll like receptor signaling pathway. Because the novel genes represent a large number of new exons, analyses for total RNA extracted from the intestinal mucosa of two NE-afflicted inbred chicken lines showing disparate immune response to NE may be a useful approach for finding novel candidate genes. These analyses indicate that major elevated expression of genes in intestinal mucosa of chicken EM/CP infected were associated with immunity and defense (cytokine-cytokine receptor interaction), signal transduction (MAPK, JAK-STAT, TGF- $\beta$ , VEGF, PPAR and Toll-like receptor signaling pathways), ECM (e.g., focal adhesion, cell adhesion and ECM-receptor interaction), and cell cycle-associated pathways such as those involved in apoptosis and cell differentiation. Most of up- and down-regulated genes involved in various immunological responses in infected specimens were likely a result of the “new” population of immune cells recruited to the infection site, rather than by the “original” population of epithelial cells. These observations were in congruence with earlier observations that interaction between immunity genes mediating and cellular immunity plays an important role in response to the coccidian infections (Lillehoj and Lillehoj, 2000; Hong et al., 2006).

We also investigated 134 immune-related genes in these two chicken lines. Overall, the expression of immune-related genes in chicken line 6.3 was higher than that in chicken line 7.2 (66 genes in line 6.3 and 56 genes in line 7.2) with  $p < 0.01$  and fold-change  $\geq 2$ . Many DEGs functioned in protein transportation, modification, and degradation. These genes are likely related to the degradation and processing of antigens for MHC class I and II molecules as shown in Table S5. Most of the DEGs were related to MHC-II antigen processing pathways, such as immunoglobulin, interferon, interleukin, heat shock protein 70 (hsp70), and hemoglobin (Table S5). Moreover, all of DEGs immune-related genes to the KEGG database for signaling pathways analysis and particularly, those genes involved in cell adhesion molecules (CAMs) pathway, inflammation mediated by chemokine and cytokine signaling pathway, integrin signaling pathway and JAK/STAT signaling pathway.

RNA-seq analysis identified 149 chicken cytokine genes that are affected by NE in these two chicken lines. This number resembles the number of such genes identified in ducks (150 genes) and zebra finches (150 genes) and is substantially lower than the number of mammalian cytokine genes, such as 230 genes in humans and 218 genes in mice (Huang et al., 2013). RNA-seq analysis provided resourceful information on the differential expression of innate immune genes or the discovery of novel genes in the intestinal mucosa in the two genetically disparate chicken lines afflicted with NE. Among the 149 innate immune genes, 37 genes were highly expressed in chicken line 6.3, and the expression of 31 genes was higher in line 7.2 than in line 6.3 (fold change  $\geq 2$ ,  $p < 0.01$ ). Cytokines are not typically stored as preformed proteins; rather, their synthesis is initiated by gene transcription following antigenic stimulation, and their mRNAs are short-lived (Kaiser et al., 2005). TNF is generally produced by activated macrophages in response to microbes, especially the lipopolysaccharide (LPS) of Gram-negative bacteria. It is an important mediator of acute inflammation (Nakagomi et al., 2010) and mediates the recruitment of neutrophils and macrophages to sites of infection by stimulating endothelial cells to produce adhesion molecules and by producing chemokines, which are chemotactic cytokines (Khawli et al., 2008). Evolutionarily, IL-10 family cytokines emerged before the adaptive immune response (Wolk et al., 2002; Alanara et al., 2010;

Xavier et al., 2013). In this study, five IL-10 family genes and 5 receptor genes were detected including IL-10, IL-19, IL-22, IL-26, IL-28B, IL-20RA, IL-20RB, IL-22RA1, IL-22RA2, and IL-28RA. The IL-20 receptor alpha (IL-20RA) gene was upregulated by 0.35 fold in line 6.3 and downregulated by 0.55 fold in chicken line 7.2 and the IL-20RB gene was upregulated in two chicken lines by 1.53–2.99 fold relative to the control with  $p < 0.01$  (Fig. 2B and Table S6). It is therefore clear that IL-10 and its family members share common receptors (Alanara et al., 2010). Often, however, cytokines have distinct, if not antagonistic, functions. As an example, both IL-10 and IL-22 signal through IL-10R2 and each activate JAK1 and TYK2, thus resulting in STAT3 activation. IL-22, however, also induces serine phosphorylation of STAT3 (IL-10 does not), an event that is associated with MAP kinase pathway activation. This notable difference in signaling is reflected in at least one function of IL-10 and IL-22. IL-10 suppresses both IL-1 and TNF-alpha production by LPS-stimulated monocytes, whereas IL-22 does not (Conti et al., 2003; Boniface et al., 2005; Sabat et al., 2007; Sabat, 2010). To confirm the RNA-Seq data, 14 innate immune genes were selected for verification by quantitative real-time RT-PCR. All differentially regulated genes examined here showed similar responses to co-infection with EM and CP in qRT-PCR and RNA-Seq. A high correlation between RNA-seq and qRT-PCR was observed, with correlation coefficients in the range of 0.85–0.89 ( $p < 0.01$ ) as shown in Fig. 5. Although the pooling of the samples obviously could have masked individual variation, our goal in the present study was to obtain a broad spectrum of chicken intestinal gene responses to NE-afflicted and to provide early insights into important pathways and processes in genetically disparate chicken lines using RNA-seq.

The results we reported in this paper will provide a foundation for more targeted future studies comparing mucosal immune responses between the two lines of chickens showing disparate NE disease susceptibility. Our study also represents the first characterization of the chicken intestinal transcriptome following NE induction and provides a comprehensive analysis of intestinal mucosa gene expression in two chicken lines that show disparate disease response to NE.

## 5. Conclusion

In summary, two chicken lines showed distinct responses to NE-afflicted. RNA-Seq analysis revealed that the number of DEGs in chicken line 6.3 was greater compared to line 7.2, and the selected transcripts were identified later in the qPCR analysis. With the reliable measurement of gene abundance in chicken intestinal mucosa by transcriptome analysis using RNA-Seq, several new pieces of evidence on how host genes might regulate the chicken-NE interaction were provided. Analysis of those DEGs suggested that many biological pathways participated in NE-afflicted which could provide important information for the clarification of the mechanism of NE disease. The significantly expressed DEGs might be considered as candidate genes for NE disease and more attention should be given for the polymorphisms of those DEGs that might be as marker-assisted sites for NE-resistant species breeding. Collectively, the results generated in this study have provided new information that our knowledge of genetic control of NE and provide a platform for detailed future analysis of NE disease resistance markers for molecular breeding.

## Sequence data availability

All raw Illumina sequence data can be obtained freely by contacting the Department of Animal Science and Technology. The AMG\_Gallus database has been uploaded in the National Agricultural Biotechnology Information Centre (NABIC, <http://nabic.rda.gov.in>).

go.kr/) [ID: NN-0915-000003, NN-0915-000004, NN-0915-000005 and NN-0915-000006].

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## Appendix A. Supplementary data

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetimm.2015.06.008>

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